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(54) Title: EUKARYOTIC INITIATION FACTOR 4E INHIBITORS AND THEIR USE AS HERBICIDES

(57) Abstract: The present invention provides for the use of compounds, including VPg, which inhibit the binding of a plant eIF4E to the cap structure of its mRNAs, as herbicides. The present invention provides for means of assaying candidate herbicides. The invention also provides candidate compounds for use as herbicides including: peptide fragments of VPg comprising eIF4E binding domains ("VPg-derived peptides"), analogs of VPg and VPg-derived peptides resulting from conservative substitutions to a naturally occurring amino acid sequence; derivatives resulting from chemical modification of VPg, VPg analogs, VPg-derived peptides and analogs of VPg-derived peptides; wherein each of the candidate compounds demonstrates the ability to inhibit the binding of plant eIF4Es to the cap structure of mRNAs thereby supporting their use as herbicides. The invention also provides for methods of preparing, formulating and delivering the herbicides.

EUKARYOTIC INITIATION FACTOR 4E INHIBITORS AND THEIR USE AS HERBICIDES

FIELD OF THE INVENTION

The invention pertains to the field of herbicides.

BACKGROUND

The eukaryotic initiation factor 4E (eIF4E) is an important translation initiation factor found in mammals, plants, and yeasts. Although most observations relating to this factor have been made in mammalian cells, the similar mechanisms of translation initiation in mammals, plants, and yeasts, and the sequence homologies of different specific proteins (Browning *et al.*, (1996) *Plant Mol. Biol.* 32:107-144), suggest that the plant eIF4E initiation factors play as important a role as their mammalian homologues.

Initiation is the rate-limiting step of translation in eukaryotes, and eIF4E has a regulatory role in this cellular event (Merrick (1992) *Microbiol. Rev.* 56:291-315; Pain *et al.*, (1996) *Eur. J. Biochem.* 236:747-771; Thach *et al.*, (1992) *Cell* 68:77-180; Hertze *et al.*, (1995) *Curr. Opin. Cell. Biol.* 7:393-398; Kaufman *et al.*, (1994) *Curr. Opin. Biotechnol.* 5:550-557). eIF4E is the least abundant of the initiation factors (De Benedetti *et al.*, (1991) *Mol. Cell. Biol.* 11:5435-5445; Rhoads *et al.*, (1993) *Prog. Nucleic Acid Res. Mol. Biol.* 46:183-219). This results in various classes of mRNAs competing with each other for translation initiation, establishing an order of priorities in the spectrum of translated mRNAs (Lodish (1974) *Nature* 251:385-388; Pelletier and Sonenberg (1987) *Biochem. Cell. Biol.* 65:576-581; Kerekatte *et al.*, (1995) *Int. J. Cancer* 64:27-31); thus, eIF4E plays an important role in the control of cellular growth (Sonenberg *et al.*, (1998) *Curr. Opin. Cell. Biol.* 10:268-275; Mader *et al.*, (1995) *Biochimie* 77:40-44).

In *Saccharomyces cerevisiae*, disruption of the gene coding for eIF4E is lethal (Altmann *et al.*, (1989) *J. Biol. Chem.* 264:12145-12147), and mutants with altered mRNA cap-binding specificity reprogram mRNA selection by ribosomes (Altmann *et al.*, (1989) *Nucleic Acids Res.* 17:5923-5931; Vasilescu *et al.*, (1996) *J. Biol. Chem.* 271:7030-7037).

In mammals, overexpression of eIF4E has been shown to transform cells in tissue culture (Lazaris-Karatzas *et al.*, (1990) *Nature* 345:544-547; De Benedetti and Rhoads (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87:8212-8216; De Benedetti *et al.*, (1994) *Mol. Cel.. Diff.* 2:347-371). Elevated eIF4E expression results in the selective increase of a few proteins

whose mRNAs are normally translationally repressed (Rhoads (1993) *J. Biol. Chem.* 268:3017-3020), including proteins important in the regulation of cell growth and differentiation and other proto-oncogenes. Examples include ornithine decarboxylase and cyclin (Shantz *et al.*, (1996) *Cancer Res.* 56:3265-3269; Rousseau *et al.*, (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93:1065-1070; Rosenwald *et al.*, (1993) *Mol. Cell Biol.* 13:7358-7363), as well as two potent cytokines, FGF-2 and VPF (Kevil *et al.*, (1995) *Oncogene* 11:2339-2348; Kevil *et al.*, (1996) *Int. J. Cancer* 65:785-790).

Just as elevated levels of eIF4E contribute to the development of a transformed cellular state, the reduction of eIF4E levels, using antisense RNA, has been shown to lengthen cell division times (De Benedetti *et al.*, (1991) *Mol. Cell. Biol.* 11:5435-5445). These *in vitro* results, emphasising the importance of eIF4E in cellular growth, have been confirmed by clinical observations. Indeed, eIF4E has been found to be uniformly elevated in human breast carcinomas but not in benign fibroadenomas or normal tissue (Kerekatte *et al.*, (1995) *Int. J. Cancer* 64:27-31; Li *et al.*, (1997) *Cancer* 79:2385-2390; Nathan *et al.*, (1997) *Oncogene* 15:1087-1094; Flynn *et al.*, (1996) *Cancer Surv.* 27:293-310). eIF4E has also been found to be elevated in head and neck squamous cell cancers (Nathan *et al.*, (1997) *Oncogene* 15(5):579-584).

The translational activity of eIF4E is controlled by an important negative regulatory mechanism in mammalian cells. 4E-binding proteins (4E-BPs) are small proteins that bind eIF4E and prevent the formation of the eIF4F complex (Pause *et al.*, (1994) *Nature* 371:762; Poulin *et al.*, (1998) *J. Biol. Chem.* 273:14002-14007; Fletcher *et al.*, (1998) *Biochemistry* 37:9-15). Normally, the eIF4E proto-oncogene must bind eIF4G in order to form the eIF4F complex. 4E-BP, however, occupies the same binding site on eIF4E as eIF4G. This competition for the eIF4E binding site has been shown to produce translation inhibition *in vitro* (Mader *et al.*, (1995) *Mol. Cell. Biol.* 15:4990; Haghighat *et al.*, (1995) *EMBO J.* 14:5701-5709). In addition, expression of 4E-BP in cells transformed following the overexpression of eIF4E has been shown to cause a significant reversion of the cell phenotype (Rousseau *et al.*, (1996) *Oncogene* 13:2415-2420). Finally, when human 4E-BP1 is expressed in a modified yeast strain in which human eIF4E is expressed instead of the endogenous yeast eIF4E [mammalian eIF4E functions in *S. cerevisiae* (Altmann *et al.*, (1989) *J. Biol. Chem.* 264:12145-12127)], growth is strongly impaired (Hughes *et al.*, (1999) *J. Biol. Chem.* 274:3261-3264).

Plants have two isomers of the cap-binding initiation factor, namely eIF(iso)4E and eIF4E (Browning *et al.*, (1987) *J. Biol. Chem.* 262:11228-11232; Browning *et al.*, (1992) *J. Biol. Chem.* 267:10096-10100). These factors play an essential role in the initiation of translation of capped mRNAs in plants. In *A. thaliana*, eIF4E and eIF(iso)4E share a 70%

identity in their amino acid sequence (Rodriguez *et al.*, (1998) *Plant J.* 13:465-473). The identity between eIF(iso)4E from *A. thaliana* and wheat is equally high at 70% (Rodriguez *et al.*, (1998) *Plant J.* 13:465-473). This high sequence homology is also found in other plant species (Browning (1996) *Plant Mol. Biol.* 32:107-144). Each isomer interacts with a specific eIF4G subunit and forms eIF4F or eIF(iso)4F respectively (Browning *et al.*, (1987) *J. Biol. Chem.* 262:11228-11232; Browning *et al.*, (1992) *J. Biol. Chem.* 267:10096-10100). The two factors are mechanistically equivalent for the translation process but exhibit differences in their ability to bind m^7GTP and other cap analogs (Browning *et al.*, (1992) *J. Biol. Chem.* 267:10096-10100), as well as in their expression in different organs (Rodriguez *et al.*, (1998)*Plant J.* 13:465-473).

The importance of eIF4E in translation regulation is further illustrated when considering that eIF4F is targeted by several animal viruses in order to take over the translation machinery for the benefit of viral mRNA translation. For instance, adeno and influenza viruses affect the phosphorylation state of eIF4E (Feigenblum and Schneide (1993) *J. Virol.* 67:3027-3035; Zhang *et al.*, (1994) *J. Virol.* 68:7040-7050). Encephalomyocarditis virus inactivates the translation factor by enhancing 4E-BP1 binding (Gingras *et al.*, (1996) *Proc. Natl. Acad. Sci. U.S.A.* 93:5578-5583). Finally, picornaviruses cleave eIF4G with the consequence that mRNAs linked to eIF4E cannot interact with 48S ribosome complexes (Haghighat *et al.*, (1996) *J. Virol.* 70:8444-8450; Svitkin *et al.*, (1999) *J. Virol.* 73:3467-3472).

Many herbicides available currently work by targeting cellular enzymes involved in the metabolism of amino acids. This means that there is a pool of amino acids that must be depleted before growth inhibition is observed. This requires a long lasting herbicide. which might have adverse effects on the environment. Additionally, plants develop detoxification mechanisms that decrease the effectiveness of these herbicides. Thus a need remains in the art for improved herbicides.

This background information is provided for the purpose of making known information believed by the applicant to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the preceding information constitutes prior art against the present invention.

SUMMARY OF THE INVENTION

The present invention provides for the use of compounds, which inhibit the binding of a plant eIF4E to the cap structure of its mRNAs, as herbicides.

The present invention also provides for the use of VPg, as a herbicide in addition to its use in an assay to determine alternative herbicides.

The present invention further provides for peptide fragments of VPg comprising eIF4E binding domains ("VPg-derived peptides") that demonstrate the ability to inhibit the binding of plant eIF4Es to the cap structure of mRNAs and their use as herbicides.

The present invention further provides for analogs of VPg, and VPg-derived peptides resulting from conservative substitutions to a naturally occurring amino acid sequence that demonstrate the ability to inhibit the binding of plant eIF4Es to the cap structure of mRNAs and their use as herbicides.

The present invention further provides for chemical derivatives resulting from chemical modification of VPg, VPg analogs, VPg-derived peptides and analogs of VPg-derived peptides that demonstrate the ability to inhibit the binding of plant eIF4Es to the cap structure of mRNAs and their use as herbicides.

The present invention provides for means of assaying candidate herbicides.

The present invention provides for methods of preparing, formulating and delivering the herbicides.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. VPg interaction with eIF4E isomers using the ELISA-based binding assay. Wells precoated with 1.0 μg of VPgPro were incubated with 2.0 μg of eIF(iso)4E from *A. thaliana* (lane 1), eIF4E from *A. thaliana* (lane 2), eIF(iso)4E from *T. aestivum* (lane 3), or no initiation factor (lane 4). In lane 5, wells were coated with BLOTTO only and incubated with 2.0 μg of eIF(iso)4E from *A. thaliana*. Complexes were detected using anti-T7 tag antibodies. Values are averages of two replicates from a typical experiment.

Figure 2. VPgPro and VPgΔPro interaction with eIF(iso)4E of *A. thaliana* using the ELISA-based binding assay. (A) Purification of VPgPro and VPgΔPro. Expression and

purification were as described in Materials and Methods. Samples were loaded onto an SDS-polyacrylamide gel as follows: 5 µg VPgPro (lane 1); and 20 µg VPgΔPro (lane 2). The gel was stained with Coomassie blue. (B) ELISA-based binding assay. Wells were coated with 1 µg of VPgPro (n) or 4 µg of VPgΔPro (l) and incubated with increasing concentrations of eIF(iso)4E from *A. thaliana*. Complexes were detected using anti-T7 tag antibodies. Values are the averages of two replicates from typical experiments.

Figure 3. Amino acid sequence of the eIF(iso)4E-binding domain of VPg and comparison with corresponding regions from other potyviruses. The sequence shown as amino acids 59 to 93 of TuMV is SEQ ID NO:1.

Figure 4. Inhibition by m^7GTP of VPg-eIF(iso)4E complex formation using the ELISA-based binding assay. (A) Wells were coated with 1 µg of VPgPro and incubated with 2 µg of eIF(iso)4E from *A. thaliana* with increasing concentrations of m^7GTP. Values are the averages of two replicates from typical experiments. (B) Lineweaver-Burk representation of binding data, 1/A *vs.* 1/[eIF(iso)4E]. Wells were coated with 1 µg of VPgPro and incubated with increasing concentrations of eIF(iso)4E from *A. thaliana* in the absence of (n) or in the presence of 0.5 µM (l) or 1.0 µM (s) m^7GTP. Values are the averages of two replicates from typical experiments. A solid line presents the best fit of the data to equation $y = ax - bx^2 + c$.

Figure 5. Schematic demonstrating the binding of VPg and m^7GTP to eIF(iso)4E.

Figure 6. VPg interaction with human and yeast eIF4E using the ELISA-based binding assay. Wells precoated with 1.0 µg of VPgPro were incubated with 2.0 µg of eIF(iso)4E from *A. thaliana* (lane 1), human eIF4E (lane 2), yeast eIF4E (lane 3) or no initiation factor was added (lane 4). In lane 5, wells were coated with BLOTTO only and incubated with 2.0 µg of eIF(iso)4E from *A. thaliana*. Complexes were detected using anti-T7 tag antibodies.

Figure 7. Immunoblot analysis of *B. perviridis* plants bombarded with TuMV plasmid cDNA. After bombardment, plants were placed in a growth chamber for 10 days. Proteins were extracted from a new leaf next to one that had been bombarded, separated on an SDS-polyacrylamide gel, transferred to a nitro-cellulose membrane, and incubated with a rabbit anti-TuMV capsid serum. Lane 1: plant bombarded with gold particles not coated with DNA; lanes 2 and 3: plants bombarded with p35Tunos; and lanes 4 to 9: plants bombarded with p35TuD77N.

Figure 8. *Arabidopsis thaliana* plants transgenic for the gene coding for VPg of TuMV. Transgenic plants were produced by *Agrobacterium*-mediated transformation. Seeds from the T1 plants were collected and screened for hygromycin resistance. 25 hygromycin-resistant seedlings were selected and T2 and T3 seeds collected. These lines were then screened for phenotypes not normally observed for non-transgenic plants. N denotes an normal, non transgenic *A. thaliana* plant. A to G are transgenic plants with atypical phenotypes.

DETAILED DESCRIPTION OF THE INVENTION

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. For purposes of the present invention, the following terms are defined below.

The term "eIF4E," as used herein, refers to eukaryotic translation initiation factor 4E and its isomers, including eIF(iso)4E and nCBP (novel cap-binding protein).

The term "herbicide," as used herein, means a composition that combats or controls undesired plant growth.

The term "VPg-derived peptides," as used herein, refers to peptides having amino acid sequences of an eIF4E binding domain of a potyviral VPg peptide, subsequences thereof, and analogs, derivatives, and variants of the sequences or subsequences thereof, which possess the ability to inhibit the formation of VPg-eIF4E complexes, to bind plant eIF4Es, and to modulate (inhibit or stimulate) binding of the cap structure of mRNAs to eIF4Es; and

The term "candidate compound" as used herein refers to analogs of VPg, VPg-derived peptides, analogs of VPg-derived peptides and chemical derivatives of VPg, analogs of VPg, VPg-derived peptides, and analogs of VPg-derived peptides. Such candidate compounds can be tested in one of the assays described herein, or within an assay known to a worker skilled in the art to determine whether it can inhibit the binding of a plant eIF4E to the cap structure of its mRNAs.

The term "herbicidal compound" as used herein describes a candidate compound or other known compound which inhibits the binding of a plant eIF4E to the cap structure of its

mRNAs. In one example, such herbicidal compounds will demonstrate the ability to inhibit the formation of VPg-eIF4E complexes with an apparent Ki of 500 μM or less.

The Use of eIF4E Inhibitors as Herbicides

The present invention provides for the use of compounds, which inhibit the binding of a plant eIF4E to the cap structure of its mRNAs, as herbicides. The herbicides of the present invention act by inhibiting translation in plants. Initiation of translation is a fundamental process and is conserved throughout the plant kingdom. It is a rate limiting step and constitutes an endpoint of gene expression. There is no alternative mechanism for the initiation of translation; it cannot be by-passed. Consequently, inhibitors of eIF4E have the potential of being very toxic; thus, low concentrations can be used, and the herbicides can be short-lived.

Targeting protein synthesis also means that herbicidal effects are immediate since there is no need to deplete amino acid pools or energy stores. This approach also has the potential of blocking the synthesis of enzymes involved in detoxification such that there is a decreased risk of developing herbicide resistance.

Not only can the herbicidal effect be immediate, it can also be transient or reversible: once the inhibitory agent is removed, protein synthesis resumes. This creates the possibility of blocking the development of certain plant organs without killing the whole plant: for instance, inhibition of flower development could be achieved for certain crop plants, such as hay.

Plant eIF4Es differ substantially from those of animals; thus, the risk of harmful effects for human beings and animals is greatly decreased. As well, eIF4Es from various plants also differ, creating the possibility of designing inhibitors that affect weed species but not crop plants.

Most herbicides target a metabolic pathway and have a deleterious consequence on plant growth (e.g. death of the plant). By targeting eIF4Es and a gene regulatory process, a spectrum of end-results can be obtained: plant development can be modulated depending on dosage. Partial inhibition or activation of eIF4E would lead to a reprogrammation of translatable mRNAs, which could have an effect on leaf and/or flower production.

One skilled in the art would appreciate the requirement that the herbicide be specific to the plant of interest and would know various means for tailoring the herbicidal composition in such a manner.

The present invention also provides methods of screening large numbers of test compounds, to identify compounds capable of inhibiting the formation of VPg-eIF4E complexes, binding plant eIF4Es, and inhibiting binding of the cap structure of mRNAs to eIF4Es, for use as herbicides or plant growth regulators.

The candidate compounds the present invention may be used as herbicides to combat or control undesired plant growth. The present invention thus describes herbicidal compositions and methods of their use.

Potential inhibitory compounds are screened from large libraries of synthetic or natural compounds. Numerous means are currently used for random and directed synthesis of saccharide, peptide, and nucleic acid based compounds. Synthetic compound libraries are commercially available from a number of companies including Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, N.J.), Brandon Associates (Merrimack, N.H.), and Microsource (New Milford, Conn.). A rare chemical library is available from Aldrich (Milwaukee, Wis.). Combinatorial libraries are available and can be prepared. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are available from, e.g., Pan Laboratories (Bothell, Wash.) or MycoSearch (North Carolina), or are readily producible. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means.

Useful inhibitory agents are identified with a range of assays employing candidate compounds or nucleic acids encoding candidate compounds. As examples, protein binding assays, nucleic acid binding assays, gel shift assays, cell-based assays, and the like are useful approaches. In one embodiment of the present invention, candidate compounds are used in *in vitro* binding assays with eIF4E. Alternatively, cell- or plant-based assays can be used to screen compounds for their abilities to inhibit the formation of VPg-eIF4E complexes, to bind plant eIF4Es, and to inhibit binding of the cap structure of mRNAs to eIF4Es.

The Use of VPg as a Herbicide

The invention provides for the use of VPg, as a herbicide. VPg is a viral-encoded protein known to be produced by potyviruses, which belong to the supergroup of "picorna-like"

viruses. The potyviral genome is a single RNA molecule of positive polarity with a poly(A) tract at its 3' end. The 5' end of the viral RNA does not have a cap structure; rather, it is covalently linked via a tyrosine residue to a viral-encoded protein termed VPg (Murphy *et al.*, (1991) *Virology* 178:285-288; Murphy *et al.*, (1996) *Virology* 220:535-538).

VPg has several suggested roles in the viral life cycle. The interaction of VPg with a viral RNA polymerase both in yeast (Hong *et al.*, (1997) *Virology* 214:159-166; Li *et al.*, (1997)) and *in vitro* (Fellers *et al.*, (1998) *J. Gen. Virol.* 79:2043-2049) supports a role in viral RNA transcription. VPg has also been implicated in overcoming plant resistance (Keller *et al.*, (1998) *Mol. Plant Microbe Interact.* 11:124-130; Masuta *et al.*, (1999) *Phytopathology* 89:118-123; Nicolas *et al.*, (1996) *Arch. Virol.* 141:1535-1552; Nicolas *et al.*, (1997) *Virology* 237:452-459; Schaad *et al.*, (1997) *J. Virol.* 71:8624-8631).

As well, VPg performs a yet-to-be-defined function in the nucleus: NIa of tobacco etch potyvirus, a precursor form of VPg, has been found in the nucleus (Carrington *et al.*, (1991) *Plant Cell* 3:953-962; Hajimorad *et al.*, (1996) *Virology* 224:368-379; Restrepo *et al.*, (1990) *Plant Cell* 2:987-998); and mutations in the VPg domain that result in the inhibition of nuclear transport debilitate genome amplification (Schaad *et al.*, (1996) *J. Virol.* 70:7039-7048). Furthermore, an interaction between turnip mosaic virus (TuMV) VPg and the translation eukaryotic initiation factor eIF(iso)4E of *Arabidopsis thaliana* has been shown (Wittmann *et al.*, (1997) *Virology* 234:84-92). TuMV is a member of the potyvirus group (Riechmann *et al.*, (1992) *J. Gen. Virol.* 73:1-16).

Thus, VPg can be used as a herbicide to inhibit binding of plant eIF4E to its cap mRNA in plants, as demonstrated in the Examples Section, below.

VPg can also be used in an assay, such as an ELISA to test for candidate herbicides, as described in the Examples section, below.

VPg Derived Peptides

The present invention further provides for peptide fragments of VPg comprising eIF4E binding domains ("VPg-derived peptides") that demonstrate the ability to inhibit the binding of plant eIF4Es to the cap structure of mRNAs and their use as herbicides.

The present invention provides novel peptides that inhibit the formation of VPg-eIF4E complexes, bind to plant eIF4Es, and inhibit binding of the eIF4Es to the cap structure of

mRNAs. The peptides are comprised of, derived from, or based on the eIF4E binding domains of VPgs ("VPg-derived peptides"). The VPg-derived peptides thereof can be defined by their abilities to inhibit the formation of VPg-eIF4E complexes and/or to inhibit binding of the cap structure of mRNAs to eIF4Es.

These modified VPg eIF4E binding domains are functionally equivalent to the VPg eIF4E binding domains. A functionally equivalent peptide is one wherein the peptide's ability to inhibit the formation of VPg-eIF4E complexes, to bind plant eIF4Es, and to inhibit binding of the cap structure of mRNAs to the eIF4Es is not substantially reduced as compared to the VPg eIF4E binding domain. These modified VPg eIF4E binding domains may be isolated or constructed through standard techniques.

In one embodiment, the VPg-derived peptides of the present invention comprise the eIF4E binding domains of potyviral VPgs. Any potyvirus may be used in this invention, including the turnip mosaic potyvirus, plum pox potyvirus, lettuce mosaic potyvirus, tobacco vein mottling potyvirus, potato mosaic potyvirus, tobacco etch potyvirus, bean common mosaic potyvirus, papaya ringspot potyvirus, and zucchini yellow mosaic potyvirus. Other potyviruses are listed in Murphy *et al.*, "Virus Taxonomy: Classification and Nomenclature of Viruses" in *Archives of Virology* Suppl. 10, (New York: Springer-Verlag Wien, 1995). All of these potyviruses, as well as any potyviruses discovered in the future, are within the scope of this invention. Preferably, the potyvirus is turnip mosaic potyvirus (TuMV).

A VPg-derived peptide may be the actual eIF4E binding domain of a potyvirus. Table I lists eIF4E binding domains of nine different potyviruses. EIF4E bindinng domains of other potyviruses may be defined by mapping as described in Example II. The eIF4E binding domains of all potyviruses are within the scope of this invention.

In an exemplary embodiment of the present invention, the VPg-derived peptide is the eIF4E binding domain of TuMV VPg. The eIF4E binding domain of TuMV VPg is a 35 amino acid sequence near the middle portion of the VPg protein, starting at amino acid position 59 and ending at amino acid position 93 (SEQ ID NO. 1).

SEQ ID NO. 1: FINMYGFDPEDFSAVRFVDPLTGATLDDNPFTDIT

The VPg-derived peptides of the present invention also include subsequences and fragments of eIF4E binding domains wherein the peptides meet the defined criteria of inhibiting the formation of VPg-eIF4E complexes, binding plant eIF4Es, and affecting eIF4E-mRNA binding.

The VPg-derived peptides of the present invention may be truncations of a VPg eIF4E binding domain, or truncations of peptides with substantial amino acid sequence identity to a VPg eIF4E binding domain. Truncations refer to the elimination of amino acid sequences from the amino terminal and/or carboxy terminal ends of the peptide. Truncations may be carried out by chemical synthesis or other known techniques, including but not limited to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, $NaBH_4$, acetylation, formylation, oxidation, reduction, and the like.

The invention also includes peptides generated by deletion of particular amino acids of a VPg eIF4E binding domain or modified VPg eIF4E binding domains while still maintaining the peptide's ability to inhibit the formation of VPg-eIF4E complexes, to bind plant eIF4Es, and to inhibit binding of the cap structure of mRNAs to eIF4Es. Deletion may be carried out by techniques known in the art.

Analogs of VPg, VPg-derived Peptides

The present invention further provides for analogs of VPg, and VPg-derived peptides resulting from conservative substitutions to a naturally occurring amino acid sequence that demonstrate the ability to inhibit the binding of plant eIF4Es to the cap structure of mRNAs and their use as herbicides.

Analogs of the VPg eIF4E binding domains are peptides having the same defined criteria of inhibiting the formation of VPg-eIF4E complexes, binding plant eIF4Es, and affecting eIF4E-mRNA binding as the VPg eIF4E binding domains, but differing in their primary amino acid sequences.

The VPg-derived peptides of the present invention may be substitution analogs, wherein an amino acid is substituted with another amino acid that does not ablate the peptide's ability to inhibit the formation of VPg-eIF4E complexes, to bind plant eIF4Es, and to inhibit binding of the cap structure of mRNAs to eIF4Es.

One skilled in the art is aware that various amino acids can be replaced in a peptide without affecting the peptide's function. Such analogs may have even higher levels of herbicidal activity than the original VPg eIF4E binding domains. For the purpose of the present invention, conservative replacements may be made between amino acids within the following groups: i) alanine, serine and threonine; ii) glutamic acid and aspartic acid;

iii) arginine and lysine; iv) asparagine and glutamine; v) isoleucine, leucine, valine and methionine; and vi) phenylalanine, tyrosine and tryptophan.

Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

Systematic substitution of one or more amino acids of a consensus sequence with a D-amino acid of the same type (e.g., D-lysine in place of L-lysine) may be used to generate more stable peptides.

Other non-naturally occurring synthetic amino acids can also be incorporated into the peptides of the present invention. Such other non-naturally occurring synthetic amino acids include those where the naturally occurring side chains of the 20 genetically encoded amino acids (or any L or D amino acid) are replaced with other side chains, for instance with groups such as alkyl, lower alkyl, cyclic 4-, 5-, 6-, to 7-membered alkyl, amide, amide lower alkyl, amide di(lower alkyl), lower alkoxy, hydroxy, carboxy and the lower ester derivatives thereof, and with 4-, 5-, 6-, to 7-membered hetereocyclic. In particular, proline analogs in which the ring size of the proline residue is changed from 5 members to 4, 6, or 7 members can be employed.

The VPg-derived peptides of the present invention may also be addition analogs, wherein the VPg peptides include additional amino acids, covalently linked to either the amino-terminal or carboxyl-terminal extent, or both, of the parent peptide of the present invention. Such additional amino acids may also include atypical amino acids.

Chemical Derivatives of VPg, VPg Analogs, VPg-Derived Peptides and Analogs Thereof

The present invention further provides for chemical derivatives resulting from chemical modification of VPg, VPg analogs, VPg-derived peptides and analogs of VPg-derived peptides that demonstrate the ability to inhibit the binding of plant eIF4Es to the cap structure of mRNAs and their use as herbicides.

Derivatives of the peptides of the invention include modifications of the amino- and carboxyl-termini and amino acid side chain chemical groups such as amines, carboxylic

acids, and alkyl and phenyl groups, to produce compounds having essentially the same activity as the unmodified peptide, and optionally having other desirable properties. For example, carboxylic acid groups of the peptide, whether carboxyl-terminal or sidechain, may be provided in the form of a salt of a pharmaceutically-acceptable cation or esterified to form a C_1 -C_{16} ester, or converted to an amide of formula NR_1R_2 wherein R_1 and R_2 are each independently H or C_1 -C_{16} alkyl, or combined to form a heterocyclic ring, such as a 5- or 6-membered ring. Amino groups of the peptide, whether amino-terminal or sidechain, may be in the form of a pharmaceutically-acceptable acid addition salt, such as the HCl, HBr, acetic, benzoic, toluene sulfonic, maleic, tartaric and other organic salts, or may be modified to C_1 -C_{16} alkyl or dialkyl amino or further converted to an amide.

Hydroxyl groups of the peptide sidechain may be converted to C_1 -C_{16} alkoxy or to a C_1 -C_{16} ester using well-recognized techniques. Phenyl and phenolic rings of the peptide sidechain may be substituted with one or more halogen atoms, such as fluorine, chlorine, bromine or iodine, or with C_1 -C_{16} alkyl, C_1 -C_{16} alkoxy, carboxylic acids and esters thereof, or amides of such carboxylic acids. Methylene groups of the peptide sidechains can be extended to homologous C_2 -C_4 alkylenes. Thiols can be protected with any one of a number of well-recognized protecting groups, such as acetamide groups.

The peptides of the present invention may also be modified by phosphorylation.

The peptides of the invention include both linear and cyclized peptides. Those skilled in the art will recognize methods for introducing cyclic structures into the peptides of this invention to select and provide conformational constraints to the structure that result in enhanced binding and/or stability. For example, a carboxyl-terminal or amino-terminal cysteine residue can be added to the peptide, so that when oxidized the peptide will contain a disulfide bond, thereby generating a cyclic peptide. Other peptide cyclizing methods include the formation of thioethers and carboxyl- and amino-terminal amides and esters.

In addition, constrained peptides may be generated by methods known in the art (Rizo and Gierasch (1992) *Ann. Rev. Biochem.* 61:387); for example, by adding internal cysteine residues capable of forming intramolecular disulfide bridges which cyclize the peptide.

In addition, VPg-derived peptides and peptide analogs of the invention may be provided as fusion proteins in which the candidate compounds are joined to the sequence of another protein or peptide either by chemical or genetic means. Fusion proteins may include either peptides joined in tandem or proteins wherein the candidate compounds are interrupted.

The candidate compounds may be fused with other candidate compounds. They may also be fused with functional targeting agents.

Assays for Herbicides

The present invention provides for means of assaying candidate herbicides. In order to be effective as a herbicide, the compound must inhibit the formation of a complex between plant eIF4E and the cap structure of plant mRNA. One exemplary method is to assay compounds for the ability to inhibit the formation of a VPg-eIF4E complex. The invention further provides efficient methods of screening large numbers of test compounds and candidate compounds, to identify agents, compounds, or lead compounds capable of inhibiting the formation of VPg-eIF4E complexes, binding plant eIF4Es, and inhibiting binding of the cap structure of mRNAs to eIF4Es, for use as herbicides.

Generally, these screening methods involve exposing a candidate compound to eIF4Es in the presence of at least one test compound, followed by identifying those test compounds that inhibit the binding of the candidate compound to eIF4E. In a preferred embodiment, a high-throughput screening protocol is used to survey a large number of test compounds for their ability to inhibit the binding of candidate compounds to eIF4E.

The methods are amenable to automated, cost-effective, high-throughput screening of chemical libraries for lead compounds. Identified compounds find use in the agricultural industries for plant trials; for example, the compounds may be derivatized and rescreened in *in vitro* and *in vivo* assays to optimize activity and minimize toxicity for agricultural development as herbicides or plant growth regulators.

A general approach to utilizing the methods of this invention involves procedures in which the binding between a candidate compound and a plant eIF4E ("binding partners") is measured in a variety of ways. One approach is to label one of the partners with an easily detectable label, place it together with the other partner in conditions under which they would normally interact, perform a separation step which separates bound labeled partner from unbound labeled partner, and then measure the amount of labeled partner bound. The effect of a test compound included in the binding reaction is determined by comparing the amount of labeled partner that binds in the presence of this agent to the amount that binds in its absence.

The separation step in this type of procedure can be accomplished in various ways. In one approach, the unlabeled partner is immobilized on a solid phase prior to the binding reaction with the labeled partner, and unbound labeled partner is removed after the binding

reaction by washing the solid phase. Attachment of the binding partner to the solid phase is accomplished in various ways known to those skilled in the art, including but not limited to chemical cross-linking, non-specific adhesion to a plastic surface, interaction with an antibody attached to the solid phase, and interaction between a ligand attached to the binding partner (such as biotin) and a ligand-binding protein (such as avidin or streptavidin) attached to the solid phase.

As an example, ELISA plates can be coated with a candidate compound using standard methods. Non-specific binding sites on the wells are then blocked. The plates are then exposed to eIF4E in the absence or presence of candidate compounds. After washings, complex formation is detected using anti-eIF4E serum.

As another example, candidate compounds can be immobilized on microtiter dishes using methods that are standard in the art. The plates are then exposed to radiolabelled eIF4E in the absence or presence of candidate compounds. Conversely, eIF4E can be immobilized, and incubated with radiolabelled Candidate compounds in the absence or presence of candidate compounds. After washing, radioactivity is detected.

Alternatively, the separation step is accomplished after the labeled partner has been allowed to interact with the unlabeled partner in solution. If the size difference between the labeled partner and the unlabeled partner permits such a separation, the separation is achieved by passing the products of the binding reaction through an ultrafilter whose pores allow passage of unbound labeled partner but not of labeled partner bound to unlabeled partner. Alternatively, the products of the binding reaction are passed through a gel filtration matrix which separates labeled partner which has bound to unlabeled partner from unbound labeled partner. This can be achieved very conveniently by choosing a gel filtration matrix whose exclusion limit is greater than the molecular size of labeled partner by itself and less than the molecular size of the complex formed by labeled partner bound to unlabeled partner; the complex passes through the gel filtration set-up in the void volume, while unbound labeled partner is eluted significantly later.

In another type of approach, separation is achieved using any reagent capable of capturing the unlabeled partner from solution, such as an antibody against the unlabeled partner or a ligand-binding protein which can interact with a ligand previously attached to this partner.

A decrease in the binding affinity of the candidate compound and the eIF4E in the presence of the test compound as compared with the binding affinity in the absence of the test compound indicates that the test compound inhibits the binding of the candidate

compound to the eIF4E. Positive "hit" compounds are those that inhibit binding of the candidate compound and eIF4E and have an apparent Ki of 500 μM or less.

In all these cases, incubations, washings, and either antibody or radioactivity detection steps can be automated, allowing for high-throughput screening of a large number of test compounds.

Compounds that inhibit eIF4E can be identified in an *in vitro* translation system that is dependent on the function of eIF4E, such as one that utilizes capped mRNAs. Proteins suitable for use as reporters in the methods of this invention include, but are not limited to, easily assayed enzymes such as β-galactosidase, luciferase, β-glucuronidase, chloramphenicol acetyl transferase, and secreted embryonic alkaline phosphatase; proteins for which immunoassays are readily available such as hormones and cytokines; proteins which confer a selective growth advantage on cells such as adenosine deaminase, aminoglycoside phosphotransferase (the product of the neo gene), dihydrofolate reductase, hygromycin-B-phosphotransferase, thymidine kinase (when used with HAT medium), xanthine-guanine phosphoribosyltransferase (XGPRT), and proteins which provide a biosynthetic capability missing from an auxotroph; proteins which confer a growth disadvantage on cells, for example enzymes that convert non-toxic substrates to toxic products such as thymidine kinase (when used with medium containing bromodeoxyuridine) and orotidine-5'-phosphate decarboxylase (when used with 5-fluoroorotic acid); and proteins which are toxic such as ricin, cholera toxin or diphtheria toxin.

Binding to plant eIF4Es and affecting binding of mRNA to plant eIF4Es may also be determined by the ability of candidate compounds to affect (inhibit or stimulate) *in vitro* translation in a wheat germ lysate. One particular *in vitro* translation assay is described. Synthetic capped RNA transcripts coding for β-glucuronidase (GUS) are produced using a standard *in vitro* transcription protocol. Wheat germ lysates commercially prepared for translation reactions (50μl) are programmed with 1 μg GUS transcripts. After 60-90 min., translation reactions are stopped by the addition of 10 volumes of GUS extraction buffer (50mM $NaPO_4$ pH7.0, 10mM 2-mercaptoethanol, 10mM Na_2EDTA, 0.1% Triton X-100). The level of GUS activity in 25 μl samples of lysate is determined by adding 25 μl of substrate buffer [2mM 4-methylumbelliferyl β-D-glucuronide (MUG) in GUS extraction buffer with 40% methanol] and incubating for 60 min at 37°C. The reactions are stopped by the addition of 0.95ml of 0.2M Na_2CO_3. Conversion of MUG to methyumbelliferone is measured with a fluorometer. Variable concentrations of candidate compounds are added

along with the GUS transcripts, and the change in the amount of GUS activity is determined. An algorithm is used for calculating the apparent inhibition constant (Ki) for the candidate compounds.

Purified recombinant eIF4E is prepared, preferably in 20mM HEPES pH7.6, 1mM DTT, 100mM KCl, and 1mM $MgCl_2$. The excitation wavelength is at 258 nm, and the emission fluorescence is monitored at 336 nm and 362 nm. Variable concentrations of candidate compounds are then added along with the eIF4E, and the change in fluorescence emitted is determined. An algorithm is used for calculating the apparent inhibition constant (Ki) for the candidate compounds.

The candidate compounds of the present invention are defined as inhibiting the formation of the complex VPg-eIF4E, binding plant eIF4Es, and affecting eIF4E-mRNA binding if they have an apparent Ki of 500 μM or less.

The specificity of the compound for the VPg-eIF4E interaction can also be assessed by examining the effect of supplementing the *in vitro* translation mixture with additional VPg and/or eIF4E. For example, if a compound interacts specifically with VPg in a manner that reduces the interaction of the VPg with eIF4E, the presence of additional VPg is expected to increase the amount of a compound required to exert the same effect on cap-dependent translation as is observed in the absence of the additional VPg. If a compound interacts specifically with eIF4E or with the interface between eIF4E and VPg in a manner that reduces the interaction of the eIF4E with VPg, the presence of additional eIF4E is expected to increase the amount of a compound required to exert the same effect on cap-dependent translation as is observed in the absence of the additional eIF4E.

The use of a capped RNA with a 5'-UTR containing significant secondary structure also enhances the specificity of translation assays relating to VPg and eIF4E, because such RNAs are highly dependent upon eIF4F (of which eIF4E is a crucial component) for translation. The use of such RNAs may therefore accentuate any effects observed on eIF4E-dependent translation. 5'-UTRs that contain significant secondary structure include, but are not limited to, the 5'-UTRs of ornithine decarboxylase and various proto-oncogenes and growth factors. A 5'-UTR with significant secondary structure is also readily generated by inserting a palindromic sequence into the DNA encoding the 5'-UTR of any mRNA. This is readily achieved by inserting multiple copies of a restriction enzyme "linker" into a site within the 5'-UTR

Cell extracts for the translation of RNAs are prepared by a variety of methods known to those skilled in the art, from sources such as rabbit reticulocytes, wheat germ, HeLa cells,

and the like. The extract may usefully be treated with micrococcal nuclease to destroy endogenous cellular mRNAs, so that RNA added to the extract is essentially the only RNA translated. Appropriate nuclease-treated extracts are available from a variety of commercial sources, such as Promega of Madison, Wis., Novagen of Madison, Wis., Life Technologies of Gaithersburg, Md., and several others.

Cap-dependent and IRES-dependent translation can also be studied in intact cells rather than cell extracts. Cells are transfected with DNA constructs using procedures well known in the art. Compounds are then tested to determine their impact on synthesis of reporter polypeptides translated via cap-dependent and IRES-dependent translation. A compound that inhibits/stimulates cap-dependent translation significantly more than IRES-dependent translation is potentially a compound that inhibits the interaction between eIF4E and VPg. Further information about the specificity of the agent is obtained by assessing its impact on translation in cells that express elevated levels of eIF4E and/or VPg as a result of being transfected with DNA constructs coding for the eIF4E and/or VPg.

In order to screen for potential herbicides or plant growth regulators, assays measuring changes in plant growth in response to test compounds may be used. Assays measuring changes in plant growth include changes in root, stem, or leaf growth. The use of transgenic plants as described in above is also included.

The following assay may be utilized in order to screen compounds for their effects on plant growth mediated by eIF4E. Arabidopsis seedlings expressing or overexpressing eIF4E are treated with the test compound. In this plant growth assay, Arabidopsis seeds are plated on tissue culture plates in MS Medium (Murashige and Skoog Salt Mixture-plant basic medium available from Gibco (BRL)). A dose-response curve is determined using various concentrations of the test compound added to the medium. The plants are grown vertically in a growth chamber at 22°C with a 16 hour light/8 hour dark cycle for two weeks. The effects of each test compound on plant growth are assessed by measuring root, stem, or leaf growth on vertical tissue culture plates. Effectiveness of the test compound is measured by an increase or reduction in root, stem, or leaf growth.

In one embodiment, inhibition of the formation of the VPg-eIF4E complex can be determined by the ability of candidate compounds such as VPg-derived peptides to inhibit the interaction of TuMV VPgs with eIF4E in an ELISA-based binding assay.

Inhibition or stimulation of mRNA binding to plant eIF4Es may also be determined by the ability of candidate compounds to inhibit the interaction of TuMV VPgs with eIF4E, since any compound that prevents VPg-eIF4E complex formation will either prevent or increase eIF4E-mRNA complex formation.

One particular ELISA-binding assay is described. Purified VPgPro in PBS(1X) is adsorbed to the wells of an ELISA plate (1 µg/well) by overnight incubation at 4 °C. The remaining binding capacity of the wells is then blocked with BLOTTO 5%/PBS1X. Purified T7tag-eIF4E, diluted in BLOTTO 1%/PBS1X with Tween 0.2%, is applied to the coated wells and incubated for 1 hr at 4°C. The amount of eIF4E binding is determined using an ELISA assay with the commercially available anti-T7tag antibody and peroxidase-labeled goat anti-mouse immunoglobulin G (KPL), as described in Example 1. Variable concentrations of candidate compounds are added along with fixed concentrations of eIF4E and the decrease in the amount of eIF4E binding is determined. An algorithm is used for calculating the apparent inhibition constant (Ki) for the candidate compounds (Cai *et al.*, (1999) *Biochemistry* 38:8538-8547).

A candidate compound is defined as inhibiting the formation of VPg-eIF4E complexes if it has an apparent Ki of 500 µM or less.

Methods of Preparing Herbicides

The present invention provides for methods of preparing the herbicides. One embodiment of the present invention provides nucleic acid molecules encoding VPg-derived peptides, or fragments thereof.

The VPg-derived peptides of the present invention may be isolated and purified from natural sources, using techniques well known in the art [See, for example, Hiebert *et al.*, (1984) *Methods Virol.* 8:225-280].

Alternatively, the candidate compounds or other herbicide compounds of the present invention may be synthesized. The VPg-derived peptides of the present invention may be chemically synthesized by any of a number of manual or automated methods of synthesis known in the art. Automated synthetic routines such as those available for use with automated peptide synthesizers are intended to come within the scope of the present invention.

The peptides can be prepared by classical methods known in the art including standard solid phase techniques. The standard methods include exclusive solid phase synthesis, partial solid phase synthesis, fragment condensation, classical solution synthesis, and recombinant DNA technology (Merrifield (1963) *J. Am. Chem. Soc.* 85:2149; Merrifield (1986) *Science* 232:341).

Once synthesized, the authenticity of the resulting peptide may be verified using known procedures, such as fast atom bombardment mass spectroscopy, amino acid sequencing, and analysis.

Isolation and recovery of the VPg-derived peptides may be effected by techniques known in the art. The peptides may be isolated and purified by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography, high performance liquid chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

The VPg-derived peptides of the present invention may also be produced by recombinant DNA technology using techniques well known in this art.

VPg-derived peptides may be synthesized using recombinant expression constructs comprising nucleic acids encoding a VPg-derived peptide. The nucleic acids encoding a VPg-derived peptide of the present invention may be obtained by screening reverse transcripts of mRNA from appropriate cells or cell line cultures, by screening genomic libraries from appropriate cells, by chemical synthesis, or by combinations of these procedures.

Screening of potyviral mRNA or genomic DNA may be carried out with oligonucleotide probes generated from the nucleic acid sequence information from the potyviral VPg eIF4E binding domains disclosed herein (Figure 3). Probes may be labeled with any detectable group and used in conventional hybridization assays.

In the alternative, nucleic acid sequences may be obtained by use of the polymerase chain reaction (PCR) procedure, using PCR oligonucleotide primers corresponding to nucleic acid sequence information derived from the potyviral VPg eIF4E binding domains disclosed herein (Figure 3). See U.S. Pat. Nos. 4,683,195 and 4,683,202.

The complete nucleic acid sequence of the TuMV RNA is disclosed in Nicolas and Laliberté (1992) *J. Gen. Virol.* 73:2785-2793.

It will be apparent to one skilled in the art that the VPg-eIF4E inhibiting activity, the eIF4E binding activity, and the eIF4E-mRNA inhibiting activity of the VPg-derived peptides disclosed herein can be created by designing a nucleic acid sequence that encodes for the eIF4E binding activity, but which differs, by reason of the redundancy of the genetic code, from the sequences disclosed herein. Accordingly, the degeneracy of the genetic code further widens the scope of the present invention as it enables major variations in the nucleotide sequence of a DNA molecule while maintaining the amino acid sequence of the encoded protein. Based upon the degeneracy of the genetic code, variant DNA sequences may be derived from the DNA sequences disclosed herein. These variant DNA sequences may be produced by modifying or synthesizing nucleic acid sequences. Variant DNA sequences include deletions, additions, or substitutions of different nucleotide residues.

Any number of techniques known in the art can be used to modify the nucleic acid sequences (See Sambrook *et al., Molecular Cloning, A Laboratory Manual* (Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory, 1989); for example, the nucleic acid sequences can be cleaved at appropriate sites with restriction endonucleases, followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. Care should be taken to ensure that the modified gene remains within the same translational reading frame as the native gene, uninterrupted by translational stop signals.

Additionally, the nucleic acid sequences can be mutated *in vitro* or *in vivo*, using standard DNA mutagenesis techniques, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including but not limited to, *in vitro* site-directed mutagenesis (Hutchinsons *et al.,* (1978) *J. Biol. Chem.* 253:6551), use of TABX linkers (Pharmacia), and like methods.

VPg-derived peptides and analogs thereof may be synthesized in host cells transformed with a recombinant expression construct comprising a nucleic acid encoding a VPg-derived peptide cDNA (See Ménard *et al.,* (1995) *Eur. J. Biochem.* 229:107-112). For the purposes of this invention, a recombinant expression construct is a replicable construct in which a nucleic acid encoding a VPg-derived peptide is operably linked to suitable control sequences capable of effecting the expression of the VPg-derived peptide in a suitable host. Generally, control sequences include a transcriptional promoter, an optional operator sequence to control transcription, sequences to control the initiation and termination of transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences to control the initiation and termination of translation (See, Sambrook *et al.,* Molecular

Cloning: A Laboratory Manual (New York: Cold Spring Harbor Press, 1989). Vectors useful for practicing the present invention include plasmids, viruses (including phage), retroviruses, and integratable DNA fragments (i.e., fragments integratable into the host genome by recombination).

A variety of host-expression vector systems may be utilized to express the VPg-derived peptide coding sequences. These include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA, or cosmid DNA expression vectors containing the VPg-derived peptide coding sequences; yeast transformed with recombinant yeast expression vectors containing the VPg-derived peptide coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the VPg-derived peptide coding sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing the VPg-derived peptide coding sequence; or animal cell systems infected with recombinant virus expression vectors (e.g., adenovirus, vaccinia virus) including cell lines engineered to contain multiple copies of the VPg-derived peptide either stably amplified (CHO/dhfr) or unstably amplified in double-minute chromosomes (e.g., murine cell lines).

The expression elements of these systems vary in their strength and specificities. Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used in the expression vector. For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage λ, plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used; when cloning in insect cell systems, promoters such as the baculovirus polyhedrin promoter may be used; when cloning in plant cell systems, promoters derived from the genome of plant cells (e.g., heat shock promoters; the promoter for the small subunit of RUBISCO; the promoter for the chlorophyll a/b binding protein) or from plant viruses (e.g., the 35S RNA promoter of CaMV; the coat protein promoter of TMV) may be used; when cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used; when generating cell lines that contain multiple copies of the VPg-derived peptides, DNA SV40-, BPV- and EBV-based vectors may be used with an appropriate selectable marker.

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the VPg-derived peptide expressed. For example, when large quantities of VPg-derived peptides are to be produced, vectors that direct the expression of high levels of fusion protein products that are readily purified may be desirable. Preferred vectors are pET21 and the like (See Ménard *et al.*, (1995) *Eur. J. Biochem.* 229:107-112). Other vectors include but are not limited to the *E. coli* expression vector pUR278 (Ruther *et al.*, (1983) *EMBO J.* 2:1791), in which the VPg-derived peptide coding sequence may be ligated into the vector in frame with the lac Z coding region so that a hybrid GluR lac Z protein is produced; pIN vectors (Inouye and Inouye (1985) *Nucleic acids Res.* 13:3101-3109; Van Heeke and Schuster (1989) *J. Biol. Chem.* 264:5503-5509); and the like. pGEX vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety. An exemplary bacterial cell line is *E. coli* strain BL21 (DE3).

In yeast, a number of vectors containing constitutive or inducible promoters may be used. For a review, see: Ausubel *et al.*, ed., *Current Protocols in Molecular Biology*, vol. 2 (Greene Publish. Assoc. & Wiley Interscience, 1988) Ch. 13; Grant *et al.*, "Expression and Secretion Vectors for Yeast" in Wu & Grossman, eds., *Methods in Enzymology*, vol. 153 (New York: Acad. Press, 1987) at 516-544; Glover, *DNA Cloning*, vol. II (Washington, DC: IRL Press, 1986) Ch. 3; Bitter, "Heterologous Gene Expression" in Berger & Kimmel, eds., *Yeast, Methods in Enzymology*, vol. 152 (New York: Acad. Press, 1987) 673-684; Strathern *et al.*, eds., *The Molecular Biology of the Yeast Saccharomyces*, vols. I and II (Cold Spring Harbor: Cold Spring Harbor Press, 1982).

In cases where plant expression vectors are used, the expression of the VPg-derived peptide coding sequences may be driven by any of a number of promoters. For example, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV (Odell *et al.*, (1985) *Nature* 313:810-812), or the coat protein promoter of TMV (Takamatsu *et al.*, (1987) *EMBO J.* 6:307-311) may be used; alternatively, plant promoters such as the small subunit of RUBISCO (Coruzzi *et al.*, (1984) *EMBO J.* 3:1671-1680; Broglie *et al.*, (1984) *Science* 224:838-843); heat shock promoters, e.g., soybean hsp17.5-E or hsp17.3-B (Gurley *et al.*, (1986) *Mol. Cell. Biol.* 6:559-565), or synthetic promoters (Gatz and Lenk (1998) *Trends Plant Sci.* 3:352-358) may be used. These constructs can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, microinjection, electroporation, etc. For reviews of such techniques see, for example,

Weissbach & Weissbach *Methods for Plant Molecular Biology* (New York: Academic Press, 1988) at 421-463; and Grierson & Corey *Plant Molecular Biology*, 2d ed. (London: Blackie, 1988) Ch. 7-9.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines that stably express the VPg-derived peptides may be engineered. Rather than using expression vectors that contain viral origins of replication, host cells can be transformed with the VPg-derived peptide DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, before being switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler *et al.*, (1977) *Cell* 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski (1962) *Proc. Natl. Acad. Sci. USA* 48:2026), and adenine phosphoribosyltransferase (Lowy *et al.*, (1980) *Cell* 22:817) genes can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler *et al.*, (1980) *Proc. Natl. Acad. Sci. USA* 77:3567; O'Hare *et al.*, (1981) *Proc. Natl. Acad. Sci. USA* 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg (1981) *Proc. Natl. Acad. Sci. USA* 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin *et al.*, (1981) *J. Mol. Biol.* 150:1); and hygro, which confers resistance to hygromycin genes (Santerre, *et al.*, (1984)*Gene* 30:147). Recently, additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:8047); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L. In: *Current Communications in Molecular Biology* (Cold Spring Harbor: Cold Spring Harbor Laboratory, 1987).

Formulations of Herbicides

The herbicidal compositions of the present invention comprise one or more herbicidal compounds as the active ingredient(s). The active ingredient(s) can be applied without

combining with other elements, or the active ingredient(s) can be prepared into a formulation. Preferably, the active ingredient(s) are mixed with a solid carrier, solvent, surfactant, and other agricultural assistants, to form an emulsion, solution, microcapsules, microemulsion, wettable powder, suspension, granules, dust, water dispersible granules, water-soluble formulation, or the like.

The solid carriers include fine powders or granules of kaolin, clay, attapulgite clay, bentonite, acid terra alba, pyrophyllite, talc, diatomaceous earth, calcite, walnut shell powder, urea, ammonium sulfate, and synthetic silicic acid hydrate.

The solvents include aromatic and aliphatic hydrocarbons such as xylene, naphthas, methylnaphthalene, paraffins, and machine oil; alcohols such as isopropanol, butanol, propylene glycol, ethylene glycol, cellosolve, and carbitol; ketones such an acetone, cyclohexanone, and isophorone; vegetable oils much as soybean oil; and cotton seed oil dimethyl sulfoxide; N,N-dimethylformamide; N-methyl pyroridone; acetonitrile; and water.

The surfactants used for emulsification, dispersion, wetting, etc. include anionic surfactants such an ligninsulfonates, polynaphthalenesulfonates, alkylsultates, alylsulfonates, alkylarylsulfonates, dialkylsulfosuccinates, polyoxyethylens alkyl ether phosphates, poiyoxyetbylene alkylaryl ether sulfates, polyoxyethylene alkylaryl other phosphates, polyoxyethylene alkylaryl ether sulfonates, as well as phosphoric and sulfuric enter salts of polyoxyethylenestyrenated and benzylated phenyl ether; and nonionic surfactants such as polyoxyethylene alkylaryl others, polyoxyethylene fatty acid esters, polyoxyethylene/polyoxypropylene block copolymers, sorbitan fatty acid esters, polyoxyethylenesorbitan fatty acid esters, as well as polyoxyethylenestyrenated and benzylated phenyl ethers.

Other formulation assistants include alginic acid salts, polyvinyl alcohols, carboxymethylcellulose, xanthane gum, and acidic isopropyl phosphate.

For further details on the formulation of crop protection compositions see, for example, G.C. Klingman. *Weed Control as a Science* (New York: John Wiley and Sons, 1961) 81-96; J.D. Freyer and S.A. Evans *Weed Control Handbook*, 5th ed. (Oxford: Blackwell Scientific Publications, 1968) 101-103.

The herbicidal compositions of the present invention may also be used with other known herbicides to further improve the herbicidal effect. In particular, it is possible to reduce the dose of the active ingredient(s) in the herbicidal compositions owing to a mixing with

other herbicides. As well, the co-application rates for the partner herbicides may correspond to or be significantly lower than recommended for use individually, due to a synergistic action among the herbicides mixed together. It is also possible to make combinations with the inventive herbicidal and a plurality of other known herbicides.

The following are examples of herbicides to be preferably associated with the herbicidal compositions of the present invention: carbamate herbicides, such as benthiocarb, molinate, and dimepiperate; thiocarbamate herbicides; acid amide herbicides, such as butachlor, pretilachlor, and mefenacet; diphenyl ether herbicides, such as chlomethoxynil and bifenox; triazine herbicides, such as atrazine and cyanazine; sulfonylurea herbicides, such as chlorsulfuron and sulfometuron-methyl; phenoxyalkanebarboxylic acid herbicides, such as MCP, and MCPB; phenoxyphenoxypropionic acid herbicides such as diclofop-methyl; pyridyloxyphenoxypropionic acid herbicides such as fluazifop-butyl; dinitroaniline herbicides, such as trifluralin and pendimethalin; urea herbicides, such as linuron and diuron; benzoylaminopropionic acid herbicides, such as benzoylprop ethyl and furanprop ethyl; imidazolinone herbicides such as imazaquin; and others, such as piperophos, dymron, bentazone, difenzoquat, naproanilide, etobenzanid, triazofenamide, quinclorac, and cyclohexanedione herticides.

The herbicidal compositions of the present invention may also be used with other compounds having biological activity, such as insecticides, acaricides, nematocides, fungicides, fertilizers, and soil improvers.

The concentration of active ingredient(s) contained in the herbicidal compositions of the present invention will vary within wide limits depending on external conditions, such as weather conditions, type of formulation, timing of the treatment, method of application, soil ecology, plants to be combatted, controlled, or regulated, and crop to be obtained. In general, however, the compositions include from 0.01 to 90% by weight of active ingredient(s) and from 0 to 20% by weight of agriculturally acceptable surfactant, the active ingredient(s) consisting of one or more candidate or herbicidal compounds. Concentrate forms of compositions generally contain between about 2 and 90%, and preferably between about 5 and 80% by weight of active ingredient(s). Application forms of formulation may for example contain from 0.01 to 20% by weight of active ingredient(s).

The present invention further comprises a method of combating or controlling undesired plant growth comprising applying to the locus where such combating or control is desired an effective amount of a herbicidal composition.

Delivering the Heribicides

The herbicides of the present invention can be delivered using a variety of methods known to one skilled in the art. The herbicidal compositions may be applied either pre- or post-emergence.

The herbicidal compositions of the present invention are applied using known application techniques, such as soil treatment before or after the germination of the weeds, foliage treatment, or flooding treatment. Soil treatment includes both soil surface treatment and soil mixing treatment. Foliage treatment includes the overall treatment of all plants and as well as local treatment in which only weeds and not crops are treated.

In one embodiment of the present invention, *Agrobacterium tumefaciens* can be employed to introduce the gene constructs into plants. Such transformations preferably use binary *Agrobacterium* Ti-DNA vectors (Bevan (1984) *Nuc. Acid Res.* 12:8711-8721), and the co-cultivation procedure (Horsch *et al.*, (1985) *Science* 227:1229-1231). Generally, the *Agrobacterium* transformation system is used to engineer dicotyledonous plants (Bevan *et al.*, (1982) *Ann. Rev. Genet.* 16:357-384; Rogers *et al.*, (1986) *Methods Enzymol.* 118:627-641). The *Agrobacterium* transformation system may also be used to transform, as well as transfer DNA to, monocotyledonous plants and plant cells (See Hernalsteen *et al.*, (1984) *EMBO J.* 3:3039-3041; Hooykass-Van Slogteren *et al.*, (1984) *Nature* 311:763-764; Grimsley *et al.*, (1987) *Nature* 325:1677-179; Boulton *et al.*, (1989) *Plant Mol. Biol.* 12:31-40.; Gould *et al.*, (1991) *Plant Physiol.* 95:426-434).

In other embodiments, various alternative methods for introducing recombinant nucleic acid constructs into plants and plant cells are utilized. These other methods are particularly useful where the target is a monocotyledonous plant or plant cell. Alternative gene transfer and transformation methods include, but are not limited to, protoplast transformation through calcium-, polyethylene glycol (PEG)- or electroporation-mediated uptake of naked DNA (see Paszkowski *et al.*, (1984) *EMBO J.* 3:2717-2722, Potrykus *et al.*, (1985) *Molec. Gen. Genet.* 199:169-177; Fromm *et al.*, (1985) *Proc. Nat. Acad. Sci. USA* 82:5824-5828; Shimamoto (1989) *Nature* 338:274-276) and electroporation of plant tissues (D'Halluin *et al.*, (1992) *Plant Cell* 4:1495-1505). Additional methods for plant cell transformation include microinjection, silicon carbide mediated DNA uptake (Kaeppler *et al.*, (1990) *Plant Cell Reporter* 9:415-418), and microprojectile bombardment (see Klein *et al.*, (1988) *Proc. Nat. Acad. Sci. USA* 85:4305-4309; Gordon-Kamm *et al.*, (1990) *Plant Cell* 2:603-618).

Physical and biochemical methods may be used to identify a plant or plant cell transformant containing the gene constructs of the present invention. These methods include but are not limited to: 1) Southern analysis or PCR amplification for detecting and determining the structure of the recombinant DNA insert; 2) Northern blot, S-1 RNase protection, primer-extension or reverse transcriptase-PCR amplification for detecting and examining RNA transcripts of the gene constructs; 3) enzymatic assays for detecting enzyme or ribozyme activity, where such gene products are encoded by the gene construct; 4) protein gel electrophoresis, western blot techniques, immunoprecipitation, or enzyme-linked immunoassays, where the gene construct products are proteins; and 5) biochemical measurements of compounds produced as a consequence of the expression of the introduced gene constructs. Additional techniques, such as in situ hybridization, enzyme staining, and immunostaining, also may be used to detect the presence or expression of the recombinant construct in specific plant organs and tissues. The methods for doing all these assays are well known to those skilled in the art.

A transformed plant cell, callus, tissue, or plant may also be identified and isolated by selecting or screening the engineered plant material for traits encoded by marker genes present on the transforming DNA. For instance, selection may be performed by growing the engineered plant material on media containing inhibitory amounts of an antibiotic to which the transforming marker gene construct confers resistance. Further, transformed plants and plant cells may also be identified by screening for the activities of any visible marker genes (e.g., the β-glucuronidase, luciferase, B or C1 genes) that may be present on the recombinant nucleic acid constructs of the present invention. Such selection and screening methodologies are well known to those skilled in the art.

The herbicides of the present invention can be expressed in transgenic plants in a tissue-specific manner: the gene coding for a VPg-derived peptide or analog can be under the control of an inducible promotor allowing precise expression of the VPg-derived peptide or analog in the appropriate tissue. The presence of the VPg-derived peptide or analog would prevent the cells of this tissue from developing normally, thus preventing the formation of this tissue. As an example, the promotor can be pollen-specific: VPg-derived peptide or analog expressed in this tissue will inhibit eIF4E and, consequently, pollen development. These transgenic plants that lack pollen will be unable to fertilize other plants.

According to the present invention, a wide variety of plants and plant cell systems may be engineered for the desired physiological and agronomic characteristics described herein using the nucleic acid constructs of the instant invention and the various transformation

methods mentioned above. In preferred embodiments, target plants and plant cells for engineering include, but are not limited to, member species of the family *Brassicaceae*, including *Brassica napus*, which produces rapeseed or canola oil; *Brassica nigra*, which produces yellow mustard; *Brassica oleracea*, whose subspecies and strains include kale and collard greens (*B. oleracea acephala*), broccoli (*B. oleracea botrytis*), cauliflower (*B. oleracea cauliflora*), head cabbage (*B. oleracea capitata*), brussel sprouts (*B. oleracea gemmifera*), and kohlrabi (*B. oleracea gongycoides*); and *Brassica rapa*, whose subspecies include pak choi (*B. rapa chinensis*), Chinese cabbage (*B. rapa pekinensis*), and turnip (*B. rapa rapifera*). Other plants are member species of the families *Solanacea* and *Gramineae*, which include *Zea mays* (corn), *Triticum aestivum* (wheat) and rice.

The present invention is described in further detail in the following non-limiting examples. It is to be understood that the examples described below are not meant to limit the scope of the present invention. It is expected that numerous variants will be obvious to the person skilled in the art to which the present invention pertains, without any departure from the spirit of the present invention. The appended claims, properly construed, form the only limitation upon the scope of the present invention.

EXAMPLES

Materials And Methods

TuMV strains:

The TuMV isolate originated from Québec, Canada and was purified as described (Nicolas and Laliberté (1991) *Journal of Virological Methods* 32:57-66). p35Tunos is a TuMV infectious cDNA clone derived from the UK1 strain (Sanchez *et al.*, (1998) *Virus Res.* 55:207-219). p35TuD77N is a p35Tunos derivative that contains the D77N mutation in the VPg domain, which abolishes the interaction of VPg with eIF(iso)4E.

Microorganisms and Media:

Manipulations of bacterial strains, yeast strains, nucleic acids, and proteins were by standard methods (Golemis *et al.*, *Current Protocols in Molecular Biology*, FM Ausubel *et al.*,eds (New York: Wiley, 1996); Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press, 1989)). *Escherichia coli* strain XL1-blue was used for subcloning, and BL21(DE3) was used for protein expression. *Saccharomyces cerivisiae* strain EGY48 (MATa, trp1, his3, ura3, 6op-Leu2) was used for the interaction study. Yeast cells were transformed by the

lithium acetate method, omitting the addition of dimethyl sulfoxide as indicated (Golemis *et al., Current Protocols in Molecular Biology,* FM Ausubel *et al.,* eds (New York: Wiley, 1996). Yeast Complete Minimal (CM) medium was used lacking specific amino acids and/or uracil (dropout), supplemented with 2% glucose or 2% galactose/ 1% raffinose (w/v).

Yeast Two-Hybrid System:

Plasmids employed for the interaction study are described in Golemis *et al., Current Protocols in Molecular Biology,* FM Ausubel *et al.,* eds (New York: Wiley, (1996). pEG202 was used for the fusion of VPg and its derivatives to the DNA-binding domain of LexA. pJG4-5 was used to express eIF(iso)4E of *A. thaliana* (pSW56) (Wittmann *et al.*, 1997) as a translation fusion to a cassette consisting of the SV40 nuclear localisation sequence, the acid blob B42, and the hemagglutinin epitope tag; expression was under the control of the GAL1 inducible promoter. The *lacZ* reporter plasmid was pSH18-34 containing eight *lexA* operators. Strength of the interaction was quantified using the β-galactosidase liquid assay (Golemis *et al., Current Protocols in Molecular Biology,* FM Ausubel *et al.,* eds (New York: Wiley, (1996)). β-galactosidase units were calculated using the following equation: units = $1000 \times (OD_{420} - 1.75 \times OD_{550})/(T \times V \times OD_{660})$ where T is time (min) and V is volume of culture used (ml).

The pLex-VPg plasmids were constructed as follows: the region coding for VPg in plasmid pET-Pro/24 (Laliberté *et al.,* (1992) *Virology* 190:510-514) was amplified by the polymerase chain reaction (PCR) using the 5′ and 3′ primer pairs listed in Table 1. The amplified fragment was digested with *BamH* I and *Xho* I, ligated with similarly restricted pEG202, and introduced into *E. coli* XL-1 and ultimately into *S. cerevisiae* EGY48. pEGVP$g_{\Delta 59\text{-}93}$ was produced by amplification of pET-Pro/24 with a first set of primers (Table 1). The amplified fragment was digested with *Eco*R I and *Xho* I and ligated with a similarly digested pKS Bluescript I (Stratagene) to produce pKS-VPg3′. Plasmid pET-Pro/24 was also amplified with a second set of primers; the amplified fragment was digested with *Bam*H I and *Eco*R I and ligated in similarly digested pKS-VPg3′. This plasmid was digested with *Bam*H I and *Xho* I and the VPg-containing fragment was ligated into *Bam*H I and *Xho* I-digested pEG202.

Recombinant protein expression in E.coli and purification:

Plasmid pETtag(iso)4E*At*, which codes for eIF(iso)4E of *A.thaliana*, was produced as follows: plasmid pSW56 was digested with *Eco*RI and *Xho*I and the 0.7 kb insert was ligated with similarly restricted pET21a (Novagen). The resulting eIF(iso)4E protein was fused at its N-terminal end to the 11 amino acid N-terminal peptide of T7 gene 10 protein, which is recognised by the anti-T7tag monoclonal antibody (Novagen).

Plasmid pETtag(iso)4E*Ta, which* codes for eIF(iso)4E of *Triticum aestivum* (wheat), was produced as follows: plasmid pGAG424/eIF(iso)4E (gift from K.S. Browning, University of Texas, USA) was digested with *Eco*RI and *Sal*I, and the encoding fragment was ligated with *Eco*RI and *Xho*I-restricted pET21a. The resulting protein was fused at its N-terminal end with the T7 tag.

Plasmid pETtag4E*At*, which codes for eIF4E of *A.thaliana,* was produced as follows: plasmid pET14b/eIF4E (gift from C. Robaglia, CEA, France) was amplified with the primers listed in Table 1; the amplified fragment was digested with enzymes *Eco*RI and *Xho*I and ligated with *Eco*RI and *Xho*I-restricted pET21a. The resulting protein was fused at its N-terminal end with the T7 tag.

The above plasmids were introduced into *E.coli* BL21 (DE3). Recombinant proteins were purified as described (Van Heerden and Browning (1994) *J. Biol. Chem.* 269:17454-17457; Wittmann *et al.*, (1997) *Virology* 234:84-92).

VPgPro was purified as previously described (Ménard *et al.*, (1995) *Eur. J. Biochem.* 229:107-112).

VPgΔPro was produced as follows: pETPro/24 and pEGVP$g_{\Delta 59\text{-}93}$ were digested with *NcoI* and *StuI*. The 5.5 kb and 0.4 kb fragments from pETPro/24 and pEGVP$g_{\Delta 59\text{-}93}$, respectively were purified and ligated. The ligation product was introduced in *E.coli* XL1 blue and ultimately in BL21(DE3). The recombinant protein was expressed and purified in the same manner as for VPgPro.

ELISA-based binding assay:

Purified VPgPro was adsorbed to the wells of an ELISA plate (1.0 µg/well) by overnight incubation at 4 °C. The remaining binding capacity of the wells was blocked with 5% BLOTTO in PBS. Purified initiation factor was diluted in 1% BLOTTO in PBS with Tween 0.2% and was incubated for 1hr at 4 °C with the previously coated wells. The detection of bound initiation factor was achieved using an ELISA assay with the anti-T7tag antibody and peroxidase labeled goat anti-mouse immunoglobulin G (KPL). Wells were washed three times with 0.05% Tween between incubations.

Site-directed mutagenesis:

PCR site-directed mutagenesis by the overlap extension method was as described (Ho *et al.*, (1989) *Gene* 77:51-59). Primers used for mutagenesis are listed in Table 1. Plasmid

p35Tunos was used as template. Amplification was performed with the Pwo DNA polymerase (Roche).

Particle bombardment:

Plasmid p35D77N was constructed as follows: p35Tunos was digested with *Cla* I and the 3.8 kb fragment was ligated with similarly digested pKS Bluescript I (Stratagene). This resulted in the recombinant plasmid pKS-Tunos/Cla. Plasmid pEG-VPgD77N was digested with *Pml I* and *Spe I*, and the corresponding fragment was inserted into pKS-Tunos/Cla linearised with *Spe I* and partially digested with *Pml I*. This last construction was digested with *Cla* I and the fragment ligated back into p35Tunos. Proper assembly was verified by nucleic acid sequencing.

Particle bombardment was done in the Biolistic PDS-1000/He instrument (BioRad). 7 µg of DNA was mixed with 3 mg of gold particles in 2.5 M $CaCl_2$ and 0.1 M spermidine. This mixture was diluted 1:5 in ethanol, and 5 µl was placed in the centre of a 900 psi rupture disk. *B. perviridis* plants at the two-leaf stage were used.

EXAMPLE 1: Interaction of TuMV VPg with eIF4E and eIF(iso)4E

Experiments were undertaken to determine if TuMV VPg interacts to both plant forms of the eukaryotic initiation factor 4E: eIF4E and eIF(iso)4E from *A. thaliana* and with eIF(iso)4E from *Triticum aestivum* (wheat). *A. thaliana* is infected by TuMV, but wheat is not.

The interactions between VPg and these initiation factors were investigated using the ELISA-based binding assay. The initiation factors were produced in *Escherichia coli* as recombinant proteins fused at their N-terminal end to the 11 amino acid N-terminal peptide of the T7 gene 10 protein (T7 tag), which is recognised by the anti-T7 tag monoclonal antibody. The proteins were purified by m^7GTP-Sepharose chromatography. ELISA plate wells were coated with 1.0 µg of recombinant VPgPro (see protein purity in Figure 2A, lane 1) and incubated with 2.0 µg of the different initiation factors. VPgPro, a precursor form of VPg, was used because it is purified more easily than VPg in *E. coli*; it has been shown that the Pro domain does not participate in eIF(iso)4E binding (Wittmann *et al.*, (1997) *Virology* 234:84-92). Complex formation was detected using anti-T7 tag antibodies. Figure 1 shows that VPgPro interacted most effectively with eIF(iso)4E of *A. thaliana* (lane 1); this level of interaction was given a relative value of 100. The initiation factor was not retained when wells were not coated with VPgPro (lane 5). Figure 1 also shows that eIF4E from *A. thaliana* (lane 2) and eIF(iso)4E from wheat

(lane 3) interacted with VPgPro. When the O.D. values were corrected for background noises [i.e. O.D. value obtained in the absence of initiation factors (lane 4)], the binding of VPgPro to eIF4E from *A. thaliana* and eIF(iso)4E from wheat was 60% and 80%, respectively, of the binding to eIF(iso)4E from *A. thaliana*.

These results show that TuMV VPg interacts with both plant forms of the eukaryotic initiation factor 4E: eIF4E and eIF(iso)4E. It also shows that complex formation takes place in many cell types and plant species.

EXAMPLE II: Mapping of the TuMV VPg Interaction Domain

The TuMV VPg domain involved in the interaction with *A. thaliana* eIF(iso)4E was mapped using the yeast two hybrid system. Deletions in the VPg gene were made by PCR and were fused to the gene coding for the DNA-binding domain of LexA in pEG202. These recombinant plasmids were introduced into yeast strain EGY48, along with either the "empty" activation domain plasmid pJG4-5, or with pSW56, which codes for eIF(iso)4E of *A. thaliana* fused to the activation domain of pJG4-5. The *lacZ* reporter plasmid pSH18-34 was also present in the yeast cells. Interaction between the different deleted VPgs and eIF(iso)4E was determined by β-galactosidase assay. The near full length VPg comprising amino acids 7 to 191 ($VPg_{7\text{-}191}$) strongly interacted with eIF(iso)4E, providing on average 659 units of β-galactosidase activity (Table 2). No activity was measured when the initiation factor was omitted. VPg deletions comprising either amino acids 7 to 63 ($VPg_{7\text{-}63}$) or amino acids 94 to 191 ($VPg_{94\text{-}191}$) failed to interact with the initiation factor. The VPg deletion comprising amino acids 62 to 191 ($VPg_{62\text{-}191}$), however, interacted strongly with eIF(iso)4E. This suggested that the region comprising amino acids 62 to 93 was involved in the interaction. To confirm this, a deletion mutant was created that lacked amino acids 59 to 93 of VPg; this deletion mutant ($VPg_{\Delta 59\text{-}93}$) did not interact with eIF(iso)4E.

To ensure that the lack of interaction with eIF(iso)4E by $VPg_{\Delta 59\text{-}93}$ was not caused by either degradation of the fusion protein or lack of nuclear transport in the yeast, an ELISA-based binding assay was performed. The deletion mutant gene was subcloned into the plasmid pET21a and expressed as a Pro fusion (VPgΔPro) in *E. coli*. The protein was purified using the same procedure as VPgPro, described above. While VPgPro was purified as a 49-kDa species (Figure 2A, lane 1), multiple forms of VPgΔPro were observed, with a main band at 46 kDa, (lane 2). This degradation of VPgΔPro suggests that deletion of the amino acids caused the protein to be more susceptible to degradation than the complete VPgPro in *E. coli*. Once purified, VPgΔPro was not susceptible to further degradation.

Conditions for the binding assay were adjusted so that similar concentrations of VPgPro and non-degraded VPgΔPro were used. ELISA plate wells were then coated with either 1.0 μg of VPgPro or 4.0 μg of VPgΔPro and incubated with increasing concentrations of eIF(iso)4E. When compared with wild-type VPgPro, VPgΔPro bound approximately five fold less initiation factor (Figure 2B). This experiment suggests that amino acids 59 to 93 of VPg are largely responsible for the binding of eIF(iso)4E.

The 35 amino acids of TuMV VPg identified above as being necessary for eIF(iso)4E binding are shown in the first line of Figure 3. (SEQ ID NO:1)

EXAMPLE III: Site-directed Mutagenesis Studies

Site-directed mutagenesis was performed to demonstrate the importance of VPg amino acids phenylalanine at position 59, tyrosine at position 63, and aspartic acid at position 77 in eIF(iso)4E binding.

TuMV infectious cDNA clone, p35Tunos, derived from the UK1 strain, (Sanchez *et al.*, (1998) *Virus Res.* 55:207-219) was used for the mutagenesis experiments so that some of the introduced mutations could be transferred back into the infectious cDNA plasmid without introducing undue changes in the nucleic acid sequence. The sequence of the VPgs from the Québec and the UK1 strains differed at several nucleic acid positions (mainly in position 3 of the codon); however, they differed by only four amino acid residues, which were clustered in the middle of the protein outside of the eIF(iso)4E binding region. The affinity of the VPgs from both strains for eIF(iso)4E was the same as determined with the yeast two-hybrid system (data not shown): the two VPgs behaved identically with respect to their interaction with the initiation factor.

PCR site-directed mutagenesis by overlap extension was used and the interactions of the mutants with eIF(iso)4E were evaluated using the yeast two-hybrid system. A portion of Pro was introduced along with VPg in pEG202 for subsequent subcloning into p35Tunos. Mutants VPg_{F59A} and VPg_{Y63A}, which introduced alanine residues at positions 59 and 63 respectively, provided similar β-galactosidase levels as the non-mutated VPg, indicating that their modification did not affect VPg interaction with the initiation factor (Table 3). Mutants VPg_{D77A}, VPg_{D77E}, and VPg_{D77N}, which introduced an alanine, glutamic acid, and asparagine at position 77 respectively, failed to interact with the translation factor. The fact that replacement of aspartic acid at position 77 with related amino acids such as glutamic acid and asparagine abolished binding stresses the importance of this residue for interaction with eIF(iso)4E.

EXAMPLE IV: Effect of m^7GTP on the formation of the VPg-eIF4E complex

eIF(iso)4E's role in the cell is to initiate assembly of the translation apparatus by binding to the 5' m^7GTP residue of mRNAs. In order to demonstrate that VPg and m^7GTP-mRNAs compete for eIF(iso)4E interaction, the influence of the cap analog m^7GTP on the formation of the VPg-eIF(iso)4E complex was tested.

ELISA plate wells were coated with 1.0 μg of recombinant VPgPro and incubated with 2.0 μg of eIF(iso)4E and various concentrations of m^7GTP. Complex formation was detected with anti-T7 tag antibodies. Figure 4A shows that increasing concentrations of the analog progressively prevents the formation of the VPg-eIF(iso)4E complex. As a control, GTP (10 μM) was used in place of the m^7GTP and found to have no effect on the formation of the complex; at this concentration, m^7GTP inhibits complex formation by 60%.

To determine whether the relationship between VPg and m^7GTP is competitive or non-competitive, ELISA plate wells were coated with 1.0 μg of recombinant VPgPro and incubated with increasing concentrations of eIF(iso)4E in the absence or presence of 0.5 and 1.0 μM m^7GTP. Binding data were treated as enzyme kinetic data and were represented as a Lineweaver-Burk plot [i. e. 1/D.O. at 492 nm vs. 1/eIF(iso)4E] (Figure 4B). The experimental points were not expected to fall on a straight line since VPg and eIF(iso)4E are in the same concentration range while in enzyme kinetics substrate concentrations are much higher than enzyme concentrations. Curves were fitted across the experimental points using least-square analysis, assuming a binomial equation of the type $y = ax - bx^2 + c$. The three lines crossed at a single point left of the y-axis. Such a pattern is indicative of mixed-type non-competitive ligand binding, meaning that eIF(iso)4E can simultaneously bind both VPg and m^7GTP, but the binding of one ligand decreases the affinity of the initiation factor for the second ligand (negative cooperativity) (I.H. Segel *Enzyme Kinetics - Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems* (New York: John Wiley & Sons, 1975)). This binding relationship is depicted in Figure 5, where K_1 and K_2 are the dissociation constants for the respective complexes, and "a" is the factor by which the constants increase when the other ligand is already bound.

Data of the type shown in Figure 4B can be used to extract the dissociation constants (Kd) for the VPg-eIF(iso)4E and m^7GTP-eIF(iso)4E complexes (I.H. Segel *Enzyme Kinetics - Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems* (New

York: John Wiley & Sons, 1975)). When 1/[eIF(iso)4E] approaches zero (i.e. [eIF(iso)4E] [VPgPro]), the bx^2 term becomes negligible and the equation is now $y = ax + c$ and has the same form as the Lineweaver-Burk equation, $1/v = Kapp/(V_{max}[S]) + 1/V_{max}$. Using the values estimated for the constants a and c for each curve, the calculated K_d for the VPg-eIF(iso)4E complex is 0.9 μM, and the K_d for m^7GTP is 0.4 μM, with the alpha factor being 4.3. The alpha factor is the factor by which the K_d of one ligand changes when the other ligand occupies eIF(iso)4E.

The fact that VPg-eIF(iso)4E complex formation is blocked by the presence of m^7GTP and that both ligands have a similar affinity for the initiation factor shows that VPg binding affects eIF(iso)4E in its capacity to interact with mRNAs.

EXAMPLE V: Specificity of VPg-peptides for plant eIF4E

To demonstrate the specificity of the VPg-peptides for plant eIF4E an ELISA-based binding assay was performed to compare VpgPro binding to eIF4E from *A. thaliana*, human and yeast. Wells were precoated with 1.0 μg of VPgPro and incubated with 2.0 μg of eIF(iso)4E from *A. thaliana* (Figure 6, lane 1), human eIF4E (Figure 6, lane 2), yeast eIF4E (Fiugre 6, lane 3) or no initiation factor (Figure 6, lane 4). A negative control was included in which wells were coated with BLOTTO only and incubated with 2.0 μg of eIF(iso)4E from *A. thaliana*. Any resulting complexes were detected using anti-T7 tag antibodies.

Figure 6 demonstrates that VPgPro bound to eIF(iso)4E from *A. thaliana*, however, no significant complex formation was observed in the wells containing either human or yeast eIF4E. This example demonstrates the specificity of VPg peptides for plant eIF4E.

EXAMPLE VI: Infection of *Brassica perviridis* plants

Plants were infected with TuMV clones to demonstrate the correlation between *in vitro* VPg-eIF(iso)4E complex formation and viral infection of whole plants.

Brassica perviridis plants were infected with p35Tunos and p35TuD77N by particle bombardment. p35D77N is a p35Tunos derivative that contains the D77N mutation in the VPg domain, which abolishes the interaction with eIF(iso)4E. After bombardment, plants were kept under an 18 hr light regime at 22 °C. After 8 days, plants bombarded with the wild-type infectious plasmid began showing initial vein clearing followed by systemic mosaic symptoms characteristic of TuMV infection. After 20 days, 14 out of the 15 plants

bombarded showed full symptoms of TuMV infection. In contrast, plants bombarded with p35TuD77N remained healthy.

The presence or absence of viral proteins was confirmed by immunoblot analysis using a rabbit anti-TuMV capsid serum (Figure 7). A strong signal of the expected molecular weight for the capsid protein was observed in plants bombarded with pTunos (lanes 2 and 3). No immunoreactive species were found in those plants bombarded with p35TuD77N (lanes 4-9). No immunoreactive signal was found in mock-bombarded plants (lane 1).

These example shows that a lack of VPg-eIF(iso)4E complex formation correlates with a lack of viral infection.

EXAMPLE VIII: VPg transgenic *A. thaliana* plants show abnormal phenotypes

The gene coding for VPg was cloned into the binary Ti vector pJO530 under the control of the enhanced cauliflower mosaic virus (CaMV) 35S promoter. pTi-VPg plasmids were introduced into *Agrobacterium tumefaciens* GV3101 (Koncz and Schell (1986) *Mol. Gen. Genet.* 204:383-396). Agrobacterium-mediated transformation was used to transfer pTi-VPg into *A. thaliana* ecotype Landsberg erecta, carrying a glabrous (*gl1*) mutation. Pots (100mm in diameter) containing 10 plants were vacuum infiltrated with *A. tumefaciens* containing pTI-VPg, according to Bechtold *et al.,* (1993) *C. R. Acad. Sci.* Ser. 3 10:1194-1199).

Seeds from the T1 plants were collected and screened for hygromycin resistance by sowing them in batches of approximately 1000 onto petri plates containing MS (Murashige and Skoog) agar plus 30 µg of hygromycin per ml. At least 25 hygromycin-resistant seedlings were selected and transferred to compost. These seedlings were grown in an environmental chamber at 22 °C under a 18h light/6h dark regime, and T2 and T3 seeds collected.

Of these plant lines, at least six showed phenotypes that were different to what is observed for a non-transformed *A. thaliana* plant. The phenotypes ranged from very slow growth, production of numerous leaves that did not grow in a rosette (normal *A. thaliana* phenotype), and/or growth of several flower shoots (Figure 8). RNA was extracted for these plants; RT-PCR analysis indicated that these plant lines harboured mRNA molecules coding for VPg.

EXAMPLE VII: eIF4e Binding Domains of Potyviral VPgs

The region of TuMV VPg necessary for eIF(iso)4E binding was mapped to a 35-amino acid stretch near the middle portion of the protein. These residues are located in a region which is conserved among potyviruses. Figure 3 shows the significant homology between the amino acid sequence of the eIF(iso)4E-binding domain of TuMV VPg and the corresponding regions from other potyviruses. Amino acid sequences were aligned using BLAST software with the BLOSUM62 matrix provided on the NCBI World Wide Web server. Numbers for TuMV represent the first and last residue positions on VPg. For the other viruses, the numbers represent the first and last residue positions on the polyprotein. Dashes indicate amino acids identical to those of the TuMV VPg. The other potyviruses are abbreviated as follows: PPV, plum pox potyvirus (accession number S47508); LMV, lettuce mosaic potyvirus (P89876); TVMV, tobacco vein mottling potyvirus (P09814); PVY, potato mosaic potyvirus (1906388); TEV, tobacco etch potyvirus (P04517); BCMV, bean common mosaic potyvirus (Q65399); PRSV, papaya ringspot potyvirus (Q01901); and ZYMV, zucchini yellow mosaic potyvirus (Q89330).

Comparison indicates that this region is highly conserved among the different potyviruses: of the 35 amino acids, twenty-one residues are identical for all or a vast majority of viruses (8 residues are identical for all listed viruses, 13 more are identical for a vast majority of listed viruses) and 7 belong to the same class. This high homology implies that potyviral VPgs, besides the TuMV protein, are equally capable of interacting with the initiation factor. The region also contains the tyrosine residue (position 63 on the TuMV VPg) that covalently links the viral RNA to VPg (Murphy *et al.*, (1991) *Virology* 178:285-288; Murphy *et al.*, (1996) *Virology* 220:535-538; Riechmann *et al.*, (1992) *J. Gen. Virol.*73:1-16).

The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

Table 1: List of oligonucleotides used in this study for plasmid construction and site-directed mutagenesis[a]

Construct	5' oligonucleotide (5'→3')	3' oligonucleotide (5'→3')
$PEGVPg_{7\text{-}191}$	AAAGGCAGGATCCAAAGACAG	AGTTACTCTCGAGGTCCACT
$PEGVPg_{94\text{-}191}$	CCATTCACGGAATTCACCCTTGTA	AGTTACTCTCGAGGTCCACT
$pEGVPg_{62\text{-}191}$	GAACAGGAGGATCCTTAACA	AGTTACTCTCGAGGTCCACT
$PEGVPg_{7\text{-}63}$	AAAGGCAGGATCCAAAGACAG	GATCAAACTCGAGCATGTTA
$pEGVPg_{\Delta 59\text{-}93}$	AAAGGCAGGATCCAAAGACAG	CATGTTAATGAATTCCCTGTTCTT
	CCATTCACGGAATTCACCCTTGTA	AGTTACTCTCGAGGTCCACT
pETeIF4E*At*	TAATTTAGGGAATTCGGAGAAACA	GCAAAGATTCTCGAGGTTTCAAGC
$pEGVPg_{F59A}$	CATGAAGCGGAATTCAAGAGGCAA	CTGACTGTTCTCGAGTGGCATTAT
	AACAGGGCAATCAACATGTAT	ATACATGTTGATTGCCTTCCTGTT
$pEGVPg_{Y63A}$	CATGAAGCGGAATTCAAGAGGCAA	CTGACTGTTCTCGAGTGGCATTAT
	TTCATCAACATGGCCGGCTTTGAT	ATCAAAGCGGGCCATGTTGATGAA
$pEGVPg_{D77A}$	CATGAAGCGGAATTCAAGAGGCAA	CTGACTGTTCTCGAGTGGCATTAT
	CGTTTCGTGGCGCCACTCACAGGA	TGTGAGTGGCGCCACGAAACG
$pEGVPg_{D77E}$	CATGAAGCGGAATTCAAGAGGCAA	CTGACTGTTCTCGAGTGGCATTAT
	CGTTTCGTGGAGCCACTCACAGGA	TCCTGTGAGTGGCTCCACGAAACG
$pEGVPg_{D77N}$	CATGAAGCGGAATTCAAGAGGCAA	CTGACTGTTCTCGAGTGGCATTAT
	CGTTTCGTGAACCCACTCACAGGA	TCCTGTGAGTGGGTTCACGAAACG

[a] Oligonucleotides on same line are used in pair and amplified fragments assembled as described in Materials and Methods

Table 2: β-galactosidase activity displayed by various VPg deletions in yeast expressing eIF(iso)4E from A. thaliana fused to the B42 activation domain

Schematic representation of VPg deletion fused to DNA binding domain of LexA	VPG RESIDUES	Interactor	β-galactosidase units
	7-191[a]	none [b]	0 [c]
		eIF(iso)4E	659
	7-63	none	6
		eIF(iso)4E	6
	94-191	none	5
		eIF(iso)4E	6
	62-191	none	9
		eIF(iso)4E	1081
	Δ59-93[d]	none	5
		eIF(iso)4E	17

[a] Numbers represent first and last residues of VPg fused to DNA binding domain of LexA;

[b] Yeast containing pJG4-5; [c] Average value of two replicates from a typical experiment;

[d] Symbol and numbers represent deleted residues on VPg_{7-191}

Table 3: β-galactosidase activity displayed by mutants of VPg in yeast expressing eIF(iso)4E from A. thaliana fused to the activation domain B42

VPg	Interactor	β-galactosidase units
Wild-type	none [a]	0 [b]
	eIF(iso)4E	178
F59A [c]	none	0
	eIF(iso)4E	125
Y63A	none	0
	eIF(iso)4E	198
D77A	none	0
	eIF(iso)4E	0
D77E	none	0
	eIF(iso)4E	0
D77N	none	0
	eIF(iso)4E	0

[a] Yeast containing pJG4-5; [b] Average value of three replicates from a typical experiment; [c] First and second letters represent original and modified residues, respectively; number is residue position on VPg

WE CLAIM:

1. Use of a compound to inhibit or control plant growth, wherein said compound inhibits binding of plant eIF4E to the cap structure of plant mRNAs.

2. The use according to claim 1, wherein said compound inhibits translation of plant protein.

3. The use according to claim 1, wherein said compound is VPg or a fragment, analog or derivative thereof.

4. A VPg-derived peptide, analog or derivative thereof, comprising an eIF4E binding domain, which inhibits binding of plant eIF4E to the cap structure of plant mRNAs.

5. The VPg-derived peptide, analog or derivative thereof, according to claim 4, wherein said eIF4E binding domain contains an amino acid sequence from a potyvirus VPg.

6. The VPg-derived peptide, analog or derivative thereof, according to claim 5, wherein said potyvirus is turnip mosaic potyvirus, plum pox potyvirus, lettuce mosaic potyvirus, tobacco vein mottling potyvirus, potato mosaic potyvirus, tobacco etch potyvirus, bean common mosaic potyvirus, papaya ringspot potyvirus, or zucchini yellow mosaic potyvirus.

7. The VPg-derived peptide, analog or derivative thereof, according to claim 6, wherein said potyvirus is turnip mosaic potyvirus

8. The VPg-derived peptide, analog or derivative thereof, according to claim 4, wherein said eIF4E binding domain comprises the amino acid sequence as set forth in SEQ ID NO:1.

9. The VPg-derived peptide, analog or derivative thereof, according to any one of claims 4, 5, 6, 7 or 8, fused to a heterologous peptide.

10. A polynucleotide encoding a VPg-derived peptide, or analog thereof, wherein said VPg-derived peptide, or analog thereof, comprises an eIF4E binding domain and inhibits binding of plant eIF4E to the cap structure of plant mRNAs.

11. A polynucleotide, or one which is substantially identical to said polynucleotide, which is complementary to the polynucleotide of claim 10.

12. The polynucleotide according to claim 10, wherein said eIF4E binding domain comprises the amino acid sequence as set forth in SEQ ID NO:1.

13. The polynucleotide according to any one of claims 10, 11 or 12, fused to a heterologous polynucleotide.

14. The polynucleotide according to any one of claims 10, 11 or 12, operably linked to a regulatory sequence that controls gene expression.

15. The polynucleotide, or one which is substantially identical to said polynucleotide, of any one of claims 10, 11 or 12, fused, in frame, to a polynucleotide sequence encoding a polypeptide which facilitates purification of the resultant fusion polypeptide.

16. A vector comprising the polynucleotide according to any one of claims 10, 11, 12, 13, 14 or 15.

17. A host cell comprising the polynucleotide, according to any one of claims 10, 11, 12, 13, 14 or 15.

18. A host cell comprising the vector according to claim 16.

19. A method for producing a VPg-derived peptide, or fragment, analog, derivative, or variant thereof, comprising:
    (a) culturing the host cell of claim 17 or 18, under conditions suitable to produce the polypeptide; and
    (b) recovering the polypeptide, or fragment, analog, derivative, or variant thereof, from the cell culture.

20. A method for producing a VPg-derived peptide, or fragment, analog, derivative, or variant thereof, comprising PCR using a VPg encoding DNA as a template and one or more primers having the nucleic acid sequence as set forth in any one of SEQ ID NOs:2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34 or 35.

21. A method of testing a compound for the ability to inhibit interaction between eIF4E and VPg, comprising:

(a) incubating VPg with eIF4E in the presence and absence of a candidate compound; and

(b) monitoring binding of the VPg to eIF4E;

wherein a decrease in binding of VPg to eIF4E in the presence of the candidate compound, in comparison to the amount of binding in the absence of the candidate compound, is indicative of inhibitory ability.

22. The method according to claim 21, wherein said VPg is immobilised.

23. A method of inhibiting plant growth comprising contacting a plant with a The VPg-derived peptide, analog or derivative thereof, according to claim 4.

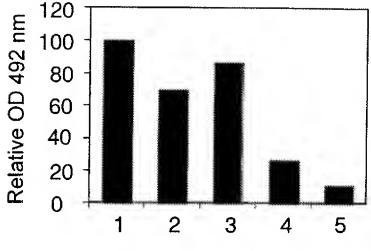

Figure 1

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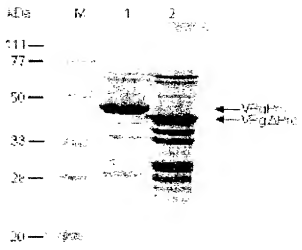
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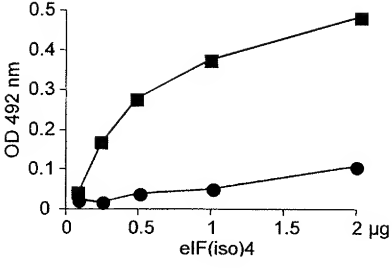
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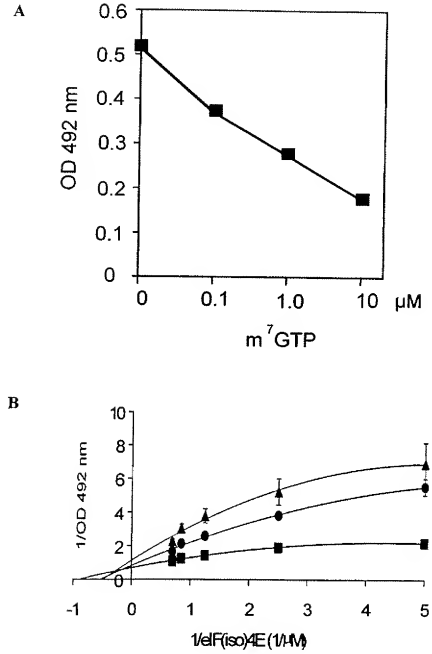
Figure 2

TuMV	59	FINMYGFDPEDFSAVRFVDPLTGATLDDNPFTDIT	93
PPV	1915	-V----Y--T-YNF---------H---E--LM--N	1949
LMV	2080	-V----YN---Y-FI--L-----K-M-EQV----S	2114
TVMV	1856	-V----VS-DEY-Y--YL--V------ES-M--LN	1890
PVY	1923	---------TEY-FIQ--------QIEE-VYA--R	1957
TEV	1907	---------T---YI--------H-I-ESSTNA-D	1941
BCMV	1920	------VE--NY-TL--------H-M-ES-RV--R	1954
PRSV	2152	-VAT---K---Y-Y--YL-----E---ES-Q---S	2186
ZYMV	1911	-VHL--VE--NY-FI--------H---ESTH---S	1945

Figure 3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μM
m7GTP
B
10
8
6
4
2
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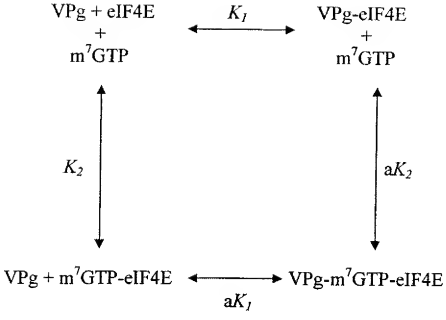
0
1
2
3
4
5
1/eIF(iso)4E (1/nM)

Figure 4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K2
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aK1
VPg-m7GTP-eIF4E

Figure 5

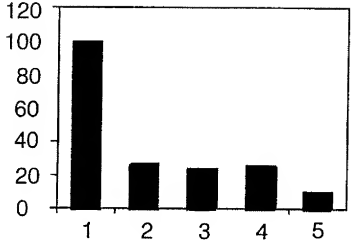

Figure 6

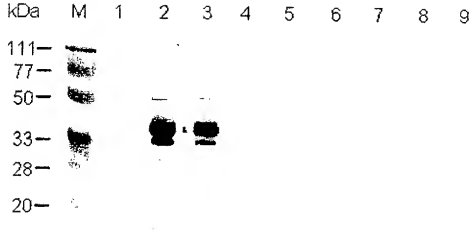

Figure 7

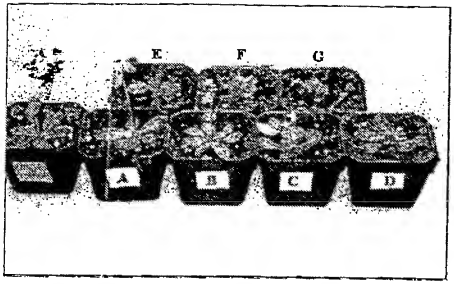

Figure 8

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Laliberte, Jean-Francois

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THEIR USE AS HERBICIDES AND PLANT GROWTH REGULATORS

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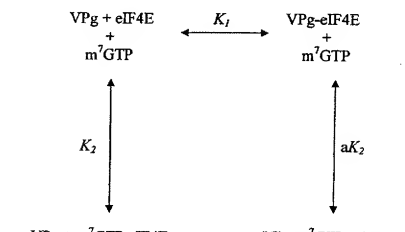
— *with international search report*

[Continued on next page]

(54) Title: EUKARYOTIC INITIATION FACTOR 4E INHIBITORS AND THEIR USE AS HERBICIDES



(57) Abstract: The present invention provides for the use of compounds, including VPg, which inhibit the binding of a plant eIF4E to the cap structure of its mRNAs, as herbicides. The present invention provides for means of assaying candidate herbicides. The invention also provides candidate compounds for use as herbicides including: peptide fragments of VPg comprising eIF4E binding domains ("VPg-derived peptides"), analogs of VPg and VPg-derived peptides resulting from conservative substitutions to a naturally occurring amino acid sequence; derivatives resulting from chemical modification of VPg, VPg analogs, VPg-derived peptides and analogs of VPg-derived peptides; wherein each of the candidate compounds demonstrates the ability to inhibit the binding of plant eIF4Es to the cap structure of mRNAs thereby supporting their use as herbicides. The invention also provides for methods of preparing, formulating and delivering the herbicides.

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— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/CA 00/01412

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C07K14/08 C12N15/62 C12Q1/68 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WITTMANN ET AL: "Interaction of the viral protein genome linked of turnip mosaic potyvirus with the translational eukaryotic initiation factor (iso) 4E of Arabidopsis thaliana using the yeast two-hybrid system" VIROLOGY, ACADEMIC PRESS,ORLANDO, US, vol. 234, 1997, pages 84-92, XP002104062 ISSN: 0042-6822 the whole document	1-23

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
3 September 2001	14/09/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Smalt, R

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INTERNATIONAL SEARCH REPORT

Int. ational Application No
PCT/CA 00/01412

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	ALTMANN M ET AL: "A novel inhibitor of cap-dependent translation initiation in yeast: p20 competes with eIF4G for binding to eIF4E" EMBO JOURNAL, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 16, no. 5, 3 March 1997 (1997-03-03), pages 1114-1121, XP002143166 ISSN: 0261-4189 the whole document	
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		EP 1056863 A	06-12-2000
US 5872011 A	16-02-1999	NONE	